

MAY 15 TRIANNUAL REPORT FOR: EFFECTS OF LYOPHILIZATION ON METABOLIC INTEGRITY OF RED BLOOD CELLS

Grant No.: N00014-94-1-0402

Performance Site: School of Pharmacy, University of Colorado Health Sciences Center

Principal Investigator: John F. Carpenter, Ph.D.

Progress reporting period: Jan. 1, 1995 - April 30, 1995 (i.e., Quarter 4 for Year 1)

I. Subprojects listed in Proposal Milestones Chart

A. Subproject A. Evaluate metabolism of fresh cells

A.1. Evaluate glycolytic flux, metabolite levels and PFK kinetic properties

Status: Work in progress, no new developments to report

A.2. Characterize enzyme binding to membrane

Status: Initiation of work delayed

B. Subproject B. Evaluate metabolism of lyophilized/rehydrated cells

B.1 and B.3 Metabolism immediately after rehydration and under simulated in vivo conditions.

Status: Work in progress

Accomplishments: We have evaluated red cells lyophilized by the most recent method from Dr. Barry Spargo's lab. The results have been compared to those for red cells obtained fresh (3 days after drawing) and those stored in liquid (Adsol) at 4°C up to the 42-day expiration date (see below). Based on heat generation rates determined by isothermal microcalorimetry, levels of ATP, ADP and AMP, and level of 2,3-DPG, the red cells that survive lyophilization and rehydration are equivalent to freshly drawn cells. And they are superior to cells that have been stored for weeks in Adsol (see below).

We will continue to collaborate with Dr. Spargo and evaluate cells prepared by improved lyophilization protocols.

B.4 Evaluate metabolism after dried storage and rehydration

Status: Work in progress, no new developments to report

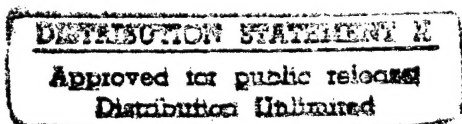
C. Subproject C. Development of quality control testing of red cells

C.1. Assess utility of HPLC for ATP and 2,3DPG assays

Status: Work in progress

Accomplishments: Another HPLC method for adenylates, which employs an aminopropyl column, was developed following published protocols. This system allows more rapid determination of adenylates, than the earlier method using a reverse phase C-18 column. Also, numerous samples can be analyzed (> 100) before column cleaning is needed. Fewer than a dozen samples could be run on the C-18 before the column had to be cleaned.

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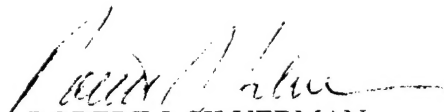
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To: Defense Technical Center, Attn: P. Mawby, 8725 John J. Kingman Rd., Suite 0944, Ft. Belvoir, VA 22060-6218

Subj: RETURNED GRANTEE/CONTRACTOR TECHNICAL REPORTS

1. This confirms our conversations of 27 Feb 97 and 11 Jul 97. Enclosed are a number of technical reports which were returned to our agency for lack of clear distribution availability statement. This confirms that all reports are unclassified and are "APPROVED FOR PUBLIC RELEASE" with no restrictions.

2. Please contact me if you require additional information. My e-mail is silverr@onr.navy.mil and my phone is (206) 625-3196.


ROBERT J. SILVERMAN

C.2. Assess utility of calorimetry for determining overall metabolic integrity.

Status: Work in progress

Accomplishments: see section B above and section E below

II. New Subprojects not listed in Milestones Chart

D. Subproject D. Determination of conditions needed to maximize ATP and 2,3-DPG levels prior to lyophilization

Status: Work in progress

Accomplishments: no work since last report

E. Subproject E. Determination of metabolic integrity of red cells stored in standard blood banking solutions

Status: Work in progress

Accomplishments:

1. A new long-term (i.e., six weeks) study of the metabolic integrity of red cells stored in liquid (Adsol) at 4°C has been completed. Cells were assessed for adenylate and 2,3-DPG levels by directly fractionating samples in Adsol. Also, cells were washed in our "physiological buffer" (see previous reports) and incubated at 37°C. Samples were removed and fractionated after 1, 4, and 24 hours at 37°C. This procedure allows assessment of metabolic status directly in Adsol and the capacity of the cells to recover metabolic function under conditions mimicking those found *in vivo*. Also, the total metabolic rate of the washed cells was tested by isothermal microcalorimetry.

We found that the metabolic heat generation rate decline each week for the first four weeks. For the remaining two weeks of the study, it remained at the same depressed level measured at week 4. These results indicate that total metabolic function was partially and irreversibly impaired in cells after 4 weeks in Adsol. Similarly, the adenylate levels measured directly in Adsol decline and shifted towards ADP and AMP, during the six-week study. The adenylate energy charge was initially about 0.9 in cells, assessed directly in Adsol, and did not change after washing and incubation at 37°C. However, it decreased to about 0.8 by week 4, with some recovery noted during post-wash incubation. By week 6, the initial value was about 0.55, with recovery to only about 0.75 noted during the course of incubation at 37°C. 2,3-DPG declined to undetectable levels by week 4 for samples assessed directly from Adsol. After this time, washing and further incubation at 37°C did not lead to a recovery of 2,3-DPG.

These results indicate that after 4 weeks of storage at 4°C in Adsol, red cells have sustained irreversible damage to metabolism. Based on earlier studies by Valeri, this damage is most likely due to the depletion of essential metabolic intermediates. He documented that if these intermediates are provided *in vitro* in a "rejuvenation" solution, then the cells can recover normal metabolic function. However, such levels of metabolic intermediates would not normally be found in the body. Thus, it appears that cells transfused after 4-6 weeks storage in Adsol (within the 42-day expiration period), would not be able to recover normal levels of adenylates, nor have the needed levels of 2,3-DPG, at least for the first 24 hours after transfusion. The former lesion could limit survival of the cells, whereas the latter could impair the capacity of the cells to deliver oxygen effectively to the tissues.

We wish to stress, however, that this long-term study has only been conducted with a single unit of blood. We are now starting a new experiment, in which

three units will be studied in parallel. Also, to determine the effects of the metabolic depression on *in vivo* survival, we have formed a collaboration with Lt. Col. Michael Fitzpatrick (U.S. Army), who is currently completing his Ph.D. thesis research at Bowling Green University. He has developed a state-of-the-art two-label fluorescent cell tagging method, which when combined with flow cytometry, allows reliable, accurate determination of red cell survival. Using rabbits, we will determine the metabolic status of red cells placed into Adsol, immediately after drawing (i.e., within 48 hours) and after 4 and 7 weeks of storage. Lt. Col. Fitzpatrick will perform survival studies in parallel.

Subproject F. Infrared spectroscopic analysis of conformational integrity of hemoglobin in lyophilized red cells

Status: Work in progress

Accomplishments:

1. We have used infrared spectroscopy to evaluate the secondary structure of hemoglobin in red cells lyophilized by Dr. Spargo's most recent protocol. As noted for cells lyophilized by earlier methods, the hemoglobin is unfolded in the dried solid, but refolds during rehydration. Many studies with purified hemoglobin (including some of our preliminary work) have indicated that the rate of formation of methemoglobin is greatly increased if the protein is unfolded. If the same relationship is true for hemoglobin in the dried red cells, then generation of methemoglobin will most likely continue as the cells are stored in the dried solid.

It may be possible to minimize this problem by developing methods to inhibit lyophilization-induced unfolding more effectively. We are investigating this approach with purified proteins. Dr. Spargo is attempting to improve stabilization of hemoglobin in the red cells by investigating methods to load cells with stabilizers that we have found to be more effective for purified proteins (e.g., trehalose).

As another approach to help mitigate this problem, we will soon begin studying means by which to maximize the activity of methemoglobin reductase in rehydrated cells. If we can take advantage of this enzyme system in the red cells, then it may be possible to reverse oxidative damage to hemoglobin that occurs during lyophilization and storage.

2. Our attempts to study alterations in red cell hemoglobin during freezing and drying by reflectance infrared spectroscopy have not been fruitful. We have reached a "Catch 22". The reflectance crystal material that we currently employ is resistant to low temperatures, but does not provide a sufficient infrared beam pathlength to make reliable measurements on intact red cells. Other materials that can provide the needed pathlength will crack at subzero temperatures.

However, we have found that high quality infrared spectra of proteins in the frozen state can be obtained with our transmission cell, which has been outfitted with a Peltier cooling device. Once we have validated the method for purified proteins, we will then test red cells formulated in Dr. Spargo's freeze-drying buffer.

Product: A manuscript describing the quantitation of lyophilization-induced alterations in protein secondary structure was published in *Journal of Pharmaceutical Sciences* 84:415-424, April, 1995. Copies of the article are enclosed. Also, abstracts describing the new method for studying proteins in the frozen state, a new analytical technique for comparing spectra globally and a method to detect subtle structural alterations in proteins with infrared spectroscopic determination of H-D exchange have been accepted for presentation

at the Annual Meeting of the American Association of Pharmaceutical Scientists. This Society provides the best forum to discuss new developments in the physical, biological and analytical aspects of lyophilization of biological systems. These abstracts will be published in the October issue of *Pharmaceutical Research*.